

Unbinding of Hyaluronan Accelerates the Enzymatic Activity of Bee Hyaluronidase^[S]

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Hyaluronan (HA), a polymeric glycosaminoglycan ubiquitously present in higher animals, is hydrolyzed by hyaluronidases (HAases). Here, we used bee HAase as a model enzyme to study the HA-HAase interaction. Located in close proximity to the active center, a bulky surface loop, which appears to obstruct one end of the substrate binding groove, was found to be functionally involved in HA turnover. To better understand kinetic changes in substrate interaction, binding of high molecular weight HA to catalytically inactive HAase was monitored by means of quartz crystal microbalance technology. Replacement of the delimiting loop by a tetrapeptide interconnection increased the affinity for HA up to 100-fold, with a K_D below 1 nM being the highest affinity among HA-binding proteins surveyed so far. The experimental data of HA-HAase interaction were further validated showing best fit to the theoretically proposed sequential two-site model. Besides the one, which had been shown previously in course of x-ray structure determination, a previously unrecognized binding site works in conjunction with an unbinding loop that facilitates liberation of hydrolyzed HA.

Hyaluronan (HA)³ is an unbranched glycosaminoglycan, which is composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, and functional roles thereof are specifically related to its polymeric size (1). The shape and function of many anatomical structures in higher organism depend on high molecular weight HA as a ubiquitously present extracellular matrix determinant (2). Moreover, long HA chains also effectively modulate immune processes (3, 4), whereas oligomeric HA stimulates angiogenesis (5), excites the synthesis of inflammatory cytokines (6), induces heat shock proteins, and suppresses apoptosis (7). The respective polymeric size is thought to be the result of the balancing action of HA synthases (8) and hyaluronidases (HAase) (9, 10), the latter being primarily responsible for HA degradation.

The HAase isolated from the venom gland of honey bee (bee venom HAase, or BVH) was the first HAase member of the endo- β -N-acetyl-hexosaminidase family (EC 3.2.1.35), to which also all human HAases belong, that could be molecularly cloned and recombinantly expressed (11). BVH is an endoglycolytic enzyme which catalyzes the hydrolysis of β -1,4 glycosidic bond of HA, as well as although with consistently lesser efficiency, chondroitin sulfates (12). In case of HA hydrolysis, the reducing end is generated at the terminal N-acetyl-D-glucosamine.

Many structural features of BVH are also present in human HAases such as HYAL1 (13) or HYAL2 (14). The 3-dimensional structure of BVH could be elucidated by means of x-ray crystallography (15). Moreover, the exact positioning of a HA tetramer tethered directly adjacent to the active center could be resolved. This data greatly argued for an acid/base catalytic mechanism, in which glutamic acid acts as the proton donor and the N-acetyl carbonyl group of the substrate as the nucleophilic base (16). The presence of an extended furrow spanning one hemisphere of the globular structure of the bee enzyme further suggested that long parts of the HA chain interact also with sites other than that next to the active center.

In this study, we used BVH as a model enzyme, primarily because we could recently establish methods for high-yield recombinant expression in *Pichia pastoris* (12). We also wanted to investigate the function of a bulky structure delimiting one end of the putative substrate binding groove in acid-active human serum hyaluronidase 1, and as recently shown by us to be involved in regulating this enzyme's activity with respect to varying pH (17). Whether structural determinants in the close vicinity to this particular structure are actually involved in regulating substrate binding, or solely influence the active center by long distance conformational changes remained elusive.

To address this question experimentally, we performed extensive kinetic analysis with wild-type recombinant BVH, as well as with a variant lacking the bulky formation. The results obtained suggest a likely role in substrate binding for the region next to the bulky formation. Working along this line we were able to reveal distinct kinetic parameters of substrate binding employing quartz crystal microbalance (QCM) technology (18, 19). Taken together, the presence of a second binding site being located opposite the face of the bulky formation could be demonstrated (Fig. 1A). This site probably controls product release.

^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables 1–3 and Figs. 1–6.

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³ The abbreviations used are: HA, hyaluronan; CS-A, chondroitin sulfate-A; CS-B, chondroitin sulfate-B; CS-C, chondroitin sulfate-C; BVH, bee venom hyaluronidase; QCM, quartz crystal microbalance.

EXPERIMENTAL PROCEDURES

Materials—Hyaluronic acid potassium salt from human umbilical cord with an estimated average molecular mass of 750 kDa (H1504, lot no. 097K1495), chondroitin sulfate A (27042, lot STBC0183V) with an estimated average molecular mass of 21 kDa (20, 21), chondroitin sulfate B (C3788, lot no. 080M1668V) of ~42 kDa (21), chondroitin sulfate C (C4384, lot no. 1426300V) of ~60 kDa (22), *N*-acetyl-glucosamine (A8625), potassium tetraborate tetrahydrate (P5754), and *p*-dimethyl-benzaldehyde (D2004) were purchased from Sigma.

Molecular Modeling—The three-dimensional structure of BVH has recently been deciphered in the presence of a HA tetrasaccharide by others (15). Three-dimensional structural models of BVH isoforms and active center variants were visualized using the open-source PyMOL 1.1eVal software (DeLano Scientific LLC) and further evaluated with the aid of the QMEAN method of SWISS-MODEL software on the basis of coordinates for BVH in conjunction with a HA tetrasaccharide (Protein Data Bank code 1FCV) to validate the degree of native-ness of the BVH isoforms compared with the wild-type structure (23).

Mutagenesis of Hyaluronidase—Numbering of the amino acids of BVH starts with Thr in its mature form (15). An inactive variant of BVH named NQ-BVH^{WT} was obtained by mutation of both amino acids in the active center, D111N and E113Q (24). The respective cDNAs were inserted into the yeast expression vector, pPIC9, in frame with the α -factor signal sequence and under the control of *AOX1* promoter using restriction sites EcoRI and NotI, respectively. Four-primer PCR technology was used to replace a 11-amino acid stretch, which loops out between the two cysteine residues at position 189 and 201 by the tetrapeptide GSGS. The mutagenesis primers were the following (the mutated nucleotides are underlined): forward, 5'-TTTCGAGGAGCCACTACCGCAATAAGGGTAGGC-3'; and reverse, 5'-TATTGCGGTAGTGGCTCCTGCGAAGCGACCACC-3'. The resulting BVH variant was called BVH^{ΔL}. This cDNA was further modified by replacement with the 5'-terminal sequence of NQ-BVH^{WT} using EcoRI and FseI endonuclease yielding a cDNA encoding for the inactive isoform NQ-BVH^{ΔL}. All sequences were validated by sequencing.

Recombinant Hyaluronidase Expression and Purification—BVH variants were expressed in *Pichia pastoris*. The pPIC9 plasmid containing either wild-type or mutated cDNA was linearized by Sall digestion and electrotransformed into GS115 strain. After methanol induction of recombinant *Pichia* strains, high yields of secreted BVH variants were found in the growth medium. The cells were removed by centrifugation, and the protein-containing supernatant was filtered through a 0.45- μ m filter and stored at -20 °C for further use.

Ion exchange chromatography was performed as described previously (12) with minor modifications. Briefly, the supernatant was diluted with 3 volumes of 50 mM sodium acetate, pH 5.0, and applied onto a cation exchange column (5-ml HiTrapTM SP HP, GE Healthcare) in a cold room. The bound enzyme was eluted by 50% salt gradient of 2 M NaCl, and subsequently, the pooled fractions were desalted on 5-ml HiTrapTM Desalting columns (GE Healthcare).

Measurement of Enzymatic Activity—Generation of reducing ends through the action of HAase was determined based on the method established by Reissig *et al.* (25). High molecular weight HA (5 mg/ml), or as a standard, *N*-acetyl-D-glucosamine were diluted to the desired concentration with 100 mM citrate buffer, pH 4.0, containing 137 mM NaCl and 2.7 mM KCl in a total volume of 100 μ l. The mixtures were prepared on ice, and after pre-incubation at 37 °C, the reaction was started by adding enzyme (15 μ g/ml of final concentration) diluted in the same buffer. The measurements were performed at 37 °C, and the reaction was stopped by heat inactivation (95 °C for 3 min). Thereafter, 20% (v/v) of 0.8 M tetraborate solution was added, and the samples were heated again at 95 °C for 3 min. After chilling on ice, 5 volumes of acetic acid containing 1% (w/v) *p*-dimethyl-benzaldehyde and 0.375% (w/v) HCl were added, and the samples were incubated at 37 °C for 20 min. The optical density was measured by spectrophotometry at 585 nm. Four to six independent experiments were performed. One unit of hyaluronidase activity is equal to the liberation of 100 nmol of *N*-acetyl-D-glucosamine reducing ends generated by 1 mg of enzyme.

Endoglycolytic cleavage within polymeric HA produces two products and thus doubles the substrate concentration. Yet, the number of β -1,4 glycolytic bonds available to the enzyme has actually decreased. Therefore, the number of cleavable β -1,4 linkages, which also equals the number of disaccharide units of a polymeric HA chain, was considered the basis for calculation of substrate concentration when building the kinetic model, also proposed previously elsewhere (26). Hence, the molar concentration of HA as a function of time is as shown in Equation 1.

$$[\text{Ch}]_t = A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) \quad (\text{Eq. 1})$$

k_1 and k_2 are rate constants for degradation, and A_1 and A_2 are concentrations of specifically accessible β -1,4 HA bonds for cleavage. The initial reaction rates were calculated as the values of the first derivative of the two exponentials at time 0. The Michaelis-Menten kinetic parameters could be calculated by non-linear regression of the data using KaleidaGraph (version 4.1.1, Synergy Software).

Quartz Crystal Microbalance Measurements—Carboxyl sensor chips were obtained from Attana AB (Stockholm, Sweden). The preparation of the sensor chips and the immobilization procedure by amine coupling were performed according to the manufacturer's instructions. Briefly, a carboxyl chip was pre-wetted with MilliQ water prior to immobilization, inserted in the Attana A100 QCM biosensor instrument (Attana AB, Stockholm, Sweden), and left to stabilize. The immobilization procedure was carried out at a flow rate of 10 μ l/min in HBS-T buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, pH 7.4) at 25 °C. Thereafter, 0.4 mM *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride and 0.1 mM Sulfo-NHS were mixed in 1:1 ratio, and the solution was injected immediately with 300 s of contact time to activate the chip surface. After rinsing the injection loop with MilliQ water the ligand solution containing 20 or 10 μ g/ml of purified NQ-BVH^{WT} and NQ-BVH^{ΔL}, respectively, in 10 mM sodium acetate buffer, pH 5.0, was loaded to the chip for 300 s. The injection loop was

washed again with MilliQ water, and finally, the remaining activated carboxyl groups on the chip surface were neutralized by the injection of 1 M ethanolamine, pH 8.5 for 300 s. For detecting background responses, a chip without protein was prepared following the same procedure. Substrate binding experiments were performed with a continuous flow (25 μ l/min) of running buffer (20 mM citrate buffer, 137 mM NaCl, 2.7 mM KCl, 0.005% Tween 20, pH 4.0) allowing for a contact time of 100 s. Substrate samples were prepared by serial dilutions in running buffer, and the interaction was recorded with the aid of Attester[®] software (Attana AB) for HA concentrations ranging from 0.05 to 1.0 mg/ml and for chondroitin sulfate concentrations ranging from 0.05 to 0.5 mg/ml injected in a random order at 37 °C. After each measurement the chip surface was regenerated by a 30-s injection of 2 M NaCl to remove any remaining analyte. Two to three independent experiments were performed.

The frequency response curves of the interaction between the various substrates at different concentrations and the immobilized inactive BVH variants, as well as the unmodified surface were analyzed by the Attache Evaluation software (version 3.3.3.1, Attana AB). The curves obtained at distinct substrate concentrations were averaged and corrected by subtracting the nonspecific binding responses obtained from measurements using an unmodified surface. The kinetic data analysis was performed using ClampXP2 (version 3.50) provided by the developers (27) by fitting theoretical models to the experimental data. First, a global analysis attempt was made, which proved insufficient. Next, a local analysis of the association and the dissociation phases was performed. The equilibrium dissociation constants of BVH variants for high molecular weight HA and chondroitin sulfates were calculated from the directly estimated association and dissociation rate constants ($K_D = k_{\text{diss}}/k_a$).

RESULTS

Enzyme Activity and Kinetic Analysis—The substrate binding groove of HAase is constrained at one end by a bulky noose, which comprises a short stretch of amino acids that loop out at a cystine bridge linking position 189 and 201. By means of computer-aided homology modeling (23), this loop could be replaced by the tetrapeptide GSGS, which yielded the enzyme variant BVH^{ΔL} (Fig. 1A). When expressed in *Pichia pastoris*, both the wild-type and the mutant form were secreted into the growth medium, from where large quantities of active enzyme could be purified (Fig. 1B). Compared with wild-type, BVH^{ΔL} exhibited lower specific activity, and although both HAases were active throughout a wide pH range, BVH^{ΔL} was found inactive above pH 7.0. For subsequent substrate binding studies, also inactive isoforms of both BVH^{WT} and BVH^{ΔL} were generated by substituting the two essential acidic residues of the active center. The resulting mutants D111N/E113Q-BVH^{WT} and D111N/E113Q-BVH^{ΔL} were named NQ-BVH^{WT} and NQ-BVH^{ΔL}; both mutant isoforms lacked any detectable HAase activity (data not shown).

Turnover rates were assessed by monitoring the formation of reducing ends during HA depolymerization. As both BVH^{WT} and BVH^{ΔL} exhibited highest activities between pH 3.0 and pH 5.5 (Fig. 1B), kinetic analyses were performed at pH 4.0. Both

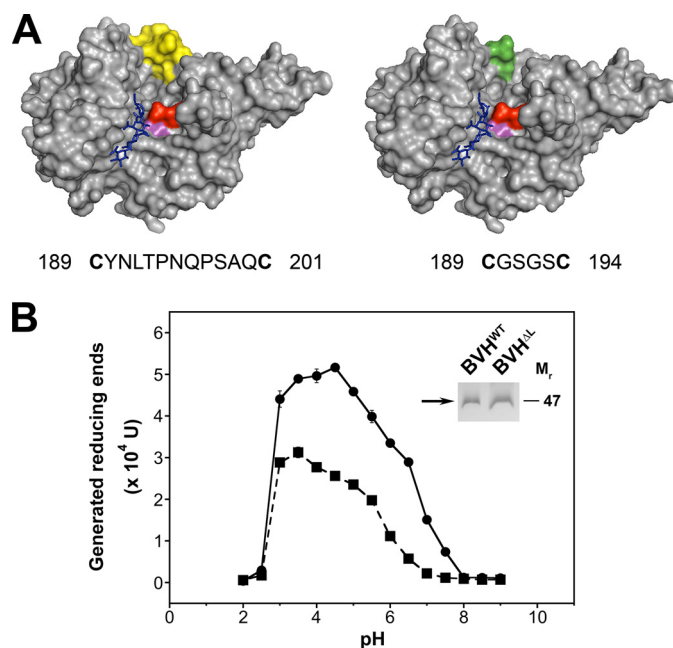


FIGURE 1. Three-dimensional models and enzymatic activity of the wild-type and the loop mutant of bee hyaluronidase. The steric models of BVH^{WT} (left) and BVH^{ΔL} (right) with a bound HA-tetrasaccharide (blue in stick mode) were generated using PyMOL. The wild-type and the mutant loops are highlighted in yellow and green, respectively, and the active centers, Asp-111 and Glu-113 are shown in magenta and red, respectively (A). Expression of the wild-type enzyme and the variant were analyzed on a 12% SDS-PAGE and visualized by Coomassie staining. The arrow indicates the expected molecular masses (inset in B). Determination of pH dependence of BVH^{WT} (filled circle and solid line), and BVH^{ΔL} (filled square and dashed line) using 0.1 mg/ml of HA concentration and 20 min of incubation time at 37 °C was performed by Reisig assay ($n = 3$). The values represent the mean \pm S.E. and in 80% of all data sets S.E. were $<10\%$. The enzyme activity is expressed in units (U), 1 unit equaling 0.1 nmol of reducing *N*-acetyl-D-glucosamine end groups generated by 1 mg of enzyme (B).

variants showed a progressive decrease of reaction rates at substrate concentrations up to 2 mg/ml. The best fit for all series of experimental data points was a biexponential function, displaying two distinct phases of a putative two-step process: during the early phase, the number of reducing ends increases rapidly, high molecular weight HA is efficiently hydrolyzed and a large amount of oligomeric substrate is generated; thereafter reaction rates gradually decrease. Even in the presence of high HA concentrations, BVH^{WT} could always reach this late phase. In contrast, BVH^{ΔL} showed reduced hydrolysis rates during the early stage, and kinetics only approached the late phase during the time course of the experiment at low HA concentrations (Fig. 2).

Next, the initial reaction rates were calculated and plotted against HA concentration. The kinetic curves clearly showed that hydrolysis rates gradually decreased at HA concentration higher than 0.5 mg/ml for BVH^{WT} or 0.2 mg/ml for BVH^{ΔL}. Consequently, the initial reaction rates showed asymmetric, bell-shaped concentration dependences with optima between 0.2–0.5 mg/ml HA for the wild-type enzyme (Fig. 3A). Despite displaying similar characteristics, the initial rate of BVH^{ΔL} was found to be 10 times lower (Fig. 3B).

The biexponential progression of HA hydrolysis could be resolved into two kinetic phases: a dominant fast-rate step (v_01) and a slow process with a distinctly lower rate (v_02). Both the

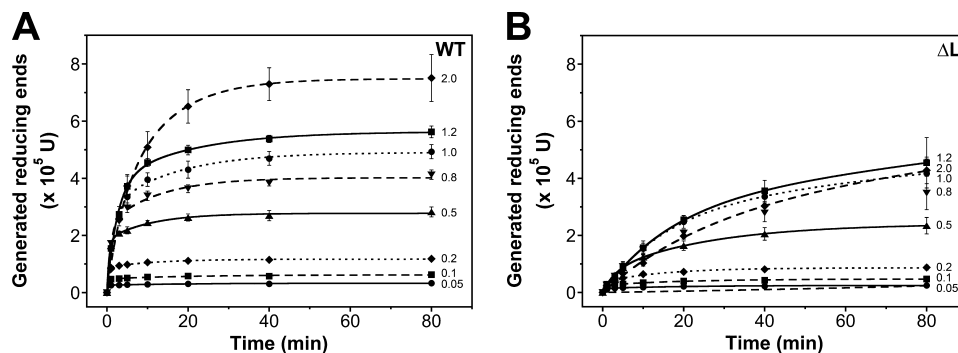


FIGURE 2. **Kinetics of HA hydrolysis at different substrate concentrations.** The time course of HA hydrolysis of BVH^{WT} (A) and BVH^{ΔL} (B) was detected by Reissig assay at pH 4.0 and at 0.05 (filled circles and solid line), 0.1 (filled squares and dashed line), 0.2 (filled diamonds and dotted line), 0.5 (filled triangles and solid line), 0.8 (filled inverted triangles and dashed line), 1.0 (filled circles and dotted line), 1.2 (filled squares and solid line), and 2.0 mg/ml (filled diamonds and dashed line) of HA concentration at 37 °C ($n = 4-6$). The enzyme activity is expressed in units (U). The values represent the mean \pm S.E. and in 90% of all data sets, S.E. were <15%.

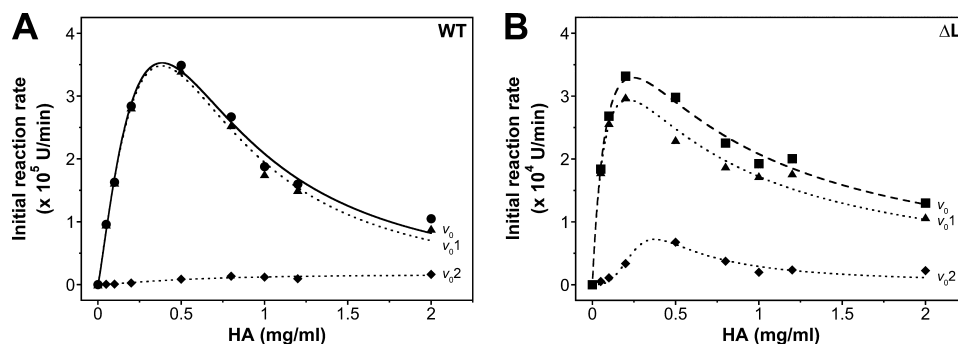


FIGURE 3. **Substrate dependence of HA hydrolysis.** Initial reaction rates of BVH^{WT} (filled circles and solid line) on A and BVH^{ΔL} (filled squares and dashed line) on B at different HA concentrations were calculated from the biexponential parameters derived from the time course measurement. Decomposing the kinetic phases yielded a fast-rate reaction (v_{01} , filled triangles and dotted line) and a slow process (v_{02} , filled diamonds and dotted line). The substrate progress curves were obtained by fitting the superposition of a saturated with an analogous displacement function.

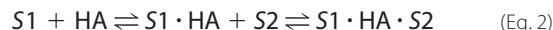
wild-type and the mutant enzyme have comparable v_{02} ; the 10-fold difference in v_0 is largely due to a rate difference of the dominant reaction v_{01} (Fig. 3). During the initial phase of the reaction, only high molecular weight HA is present, which is degraded by the wild-type enzyme with high v_{01} . This suggests that the turnover rate of long chains appears to be enhanced through the action of the bulky loop, be it through long distance conformational changes of the active center or modulated through influencing substrate binding.

Next, V_{max} and K_m of the hydrolysis reaction were estimated by performing curve fitting of the ascending and descending phases of the initial rate progress. The V_{max} for BVH^{WT} was $1 \cdot 10^6 \pm 3 \cdot 10^4$ and that of BVH^{ΔL} converged to $1.3 \cdot 10^5 \pm 2 \cdot 10^3$ units/min. Unexpectedly, BVH^{ΔL} had a lower K_m compared with BVH^{WT} (0.24 ± 0.01 versus 0.37 ± 0.08 mg/ml), indicative for higher substrate affinity.

Substrate Binding—Under the experimental conditions used in the previous kinetic analyses, substrate degradation rates of BVH and the loop variant were too high for binding studies. Therefore, interactions at varying HA concentrations (0.05–1 mg/ml) were studied with the inactive HAase variants NQ-BVH^{WT} and NQ-BVH^{ΔL}. Both HAase variants firmly bound high molecular weight HA. The point mutations introduced in the active center, although located within the substrate binding groove did not induce major conformational changes, which incommode HA binding. Moreover, the polymer could be detached from both variants. Prior to a successive

measurement, the chip was efficiently regenerated by washes with high ionic strength buffer (Fig. 4).

Working along these lines, experimental data for HA association to and dissociation from the inactive HAase variants were collected. The results obtained thereby were further validated using previously established theoretical models. A fit to the simple Langmuir binding model could not be accomplished, both the simple binding, as well as the mass transport-limited models were unable to adequately describe the interaction. This suggested that interactions between HA and BVH are more complex. Therefore, various two-state models were challenged (28–30), and finally, a two-site model was computed, which provided useful fits. When supposing substrate binding in random order, the estimation of two out of four association rate constants (k_a) yielded no meaningful results, suggesting that distinct binding events are either negligible or random combinations of binding and unbinding are likely circumstantial. Challenging the alternative proposition that binding may take place in a directed fashion the following sequential two-site model was fitted (Equation 2).



S1 and S2 are putative substrate binding sites, and thus, this model assumed that the enzyme actually accommodates two distinct binding sites, which associate with the polymer in a sequential order. Also, this particular model approximated the

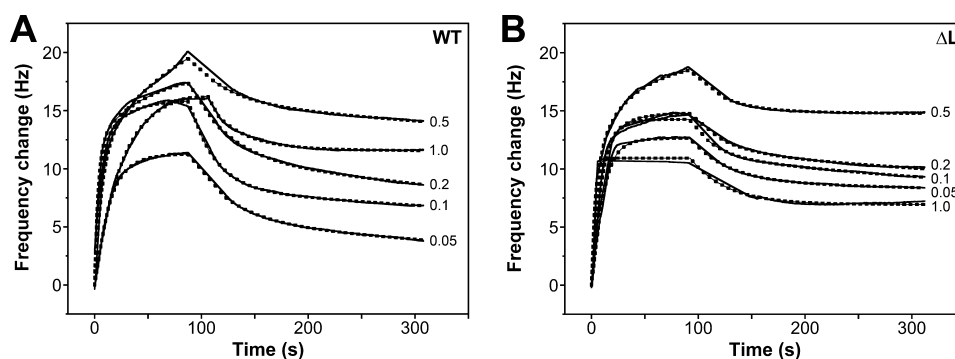


FIGURE 4. **QCM sensorgrams for substrate binding.** Inactive forms of BVH containing the normal (A) and the mutated loop sequences (B) were used for QCM measurements. Varying HA concentrations (0.05 to 1.0 mg/ml) were loaded onto immobilized BVH in a random order, and interactions were monitored at pH 4.0 and at 37 °C (solid lines) ($n = 2-3$). The curves from each HA concentrations were averaged and corrected by subtracting the nonspecific binding responses obtained from the unfunctionalized surface. The sequential two-site model was fitted (dotted lines) to the averaged sensorgrams by local analysis using ClampXP2 software.

TABLE 1

Kinetic parameters of HA binding determined by QCM approach

The kinetic data were evaluated using a two-state (sequential two-site) model, and the kinetic parameters (mean \pm S.E.) were calculated based on the average molecular mass of 750 kDa for HA.

			HA concentration (mg/ml)				
Interaction phase		Kinetic parameter	0.05	0.1	0.2	0.5	1.0
NQ-BVH ^{WT}							
Equilibrium	K_{D1} ($\times 10^{-8}$)	M	3.22 \pm 0.12	3.05 \pm 0.32	2.48 \pm 0.16	9.84 \pm 0.61	87.7 \pm 2.76
	K_{D2} ($\times 10^{-2}$)		5.48 \pm 1.13	12.0 \pm 3.70	52.0 \pm 8.71	64.0 \pm 17.6	0.69 \pm 0.35
	K_{DSUM} ($\times 10^{-9}$)		1.77 \pm 0.43	3.66 \pm 1.51	12.9 \pm 3.00	62.9 \pm 21.1	6.10 \pm 3.26
Association	k_{a1} ($\times 10^4$)	M ⁻¹ ·s ⁻¹	66.7 \pm 1.52	99.2 \pm 4.72	106 \pm 2.69	18.5 \pm 0.39	3.34 \pm 0.04
	k_{a2} ($\times 10^{-3}$)		54.9 \pm 4.95	13.5 \pm 1.89	2.64 \pm 0.24	0.78 \pm 0.14	8.62 \pm 0.31
	k_{aSUM} ($\times 10^3$)		36.6 \pm 4.14	13.4 \pm 2.51	2.80 \pm 0.33	0.14 \pm 0.03	0.29 \pm 0.01
Dissociation	k_{diss1} ($\times 10^{-3}$)	s ⁻¹	21.5 \pm 0.34	30.3 \pm 1.72	26.3 \pm 1.04	18.2 \pm 0.74	29.3 \pm 0.57
	k_{diss2} ($\times 10^{-4}$)		30.1 \pm 3.50	16.5 \pm 2.78	13.7 \pm 1.05	5.06 \pm 0.48	0.00 ^(a) \pm 0.28
	$k_{dissSUM}$ ($\times 10^{-6}$)		64.7 \pm 8.55	50.0 \pm 11.3	36.0 \pm 4.19	9.21 \pm 1.25	1.76 \pm 0.85
NQ-BVH ^{ΔL}							
Equilibrium	K_{D1} ($\times 10^{-8}$)	M	2.40 \pm 0.27	4.69 \pm 0.49	2.59 \pm 0.29	6.34 \pm 0.55	12.5 \pm 6.63
	K_{D2} ($\times 10^{-2}$)		0.48 \pm 0.09	0.91 \pm 0.54	3.80 \pm 1.03	1.11 \pm 0.59	0.04 \pm 0.03
	K_{DSUM} ($\times 10^{-9}$)		0.11 \pm 0.03	0.42 \pm 0.29	0.98 \pm 0.37	0.70 \pm 0.44	0.05 \pm 0.07
Association	k_{a1} ($\times 10^4$)	M ⁻¹ ·s ⁻¹	108 \pm 9.06	78.3 \pm 2.37	80.9 \pm 2.55	37.3 \pm 0.77	23.2 \pm 10.3
	k_{a2} ($\times 10^{-3}$)		62.0 \pm 2.02	42.4 \pm 15.4	9.21 \pm 0.58	5.41 \pm 0.27	150 \pm 30.9
	k_{aSUM} ($\times 10^3$)		67.0 \pm 7.80	33.2 \pm 13.1	7.45 \pm 0.70	2.01 \pm 0.14	34.8 \pm 22.6
Dissociation	k_{diss1} ($\times 10^{-3}$)	s ⁻¹	25.9 \pm 0.69	36.7 \pm 2.72	20.9 \pm 1.65	23.6 \pm 1.58	29.0 \pm 2.51
	k_{diss2} ($\times 10^{-4}$)		2.99 \pm 0.48	3.84 \pm 0.89	3.50 \pm 0.73	0.00 ^a \pm 0.29	0.00 ^a \pm 0.37
	$k_{dissSUM}$ ($\times 10^{-6}$)		7.74 \pm 1.45	14.1 \pm 4.31	7.32 \pm 2.10	1.42 \pm 0.78	1.74 \pm 1.22

^a ClampXP2 cannot calculate k_{diss} values $< 10^{-4}$; therefore, instead of zero, an estimated value of 0.6×10^{-4} was substituted.

experimental data regarding the interaction of HA with the two inactive variants best, as the kinetics showed congruent matches for all experimental data sets (Fig. 4). Because the global fitting algorithm could not be applied, the association (k_a) and dissociation (k_{diss}) constants, as well as the equilibrium dissociation constants (K_D) were determined for each HA concentration by local fitting (Table 1).

When plotting k_a and k_{diss} rate constants of the two binding sites against substrate concentration, it became apparent that when comparing the derived data with that of the wild-type enzyme, the inactive loop variant, NQ-BVH^{ΔL} formed the HA-enzyme complex at a considerably higher rate as reflected by a higher k_a , at the second binding site. Moreover, HA was released more slowly from the complex as k_{diss} at the second binding site was distinctly lower. In either variant, k_a and k_{diss} of the first binding site were found comparable, thus indicating that the putative second binding site is locally close to the bulky loop (Fig. 5, A and B). Also, line up of the K_D regarding the first binding sites (K_{D1}) showed highly similar results for both variants, with a sole exception of a slight difference at the highest

experimental HA concentration. In the case of NQ-BVH^{ΔL}, the second binding site exhibited again a significantly lower K_{D2} (Fig. 5C), by and large confirming the aforementioned assumption that the bulky formation solely impinges on the putative second binding site, which has high HA affinity when not affected by the loop.

Furthermore, comparison of the overall affinity constants (K_{DSUM}) as calculated from the K_D values of both binding site ($K_{D1} \times K_{D2}$), revealed the highest value for the wild-type protein with 62.9 nM at 0.5 mg/ml HA, whereas the loop variant exhibited 10 to 100 times lower K_D values with a maximum value of 0.98 nM at 0.2 mg/ml HA concentration (Table 1). Thus, replacement of the bulky formation greatly enhanced the overall affinity of the enzyme for high molecular weight HA yielding tight binding and/or impeded product release.

Chondroitin sulfate (CS) A, B, and C are also substrates to BVH. Compared with HA, the hydrolysis rate of CS-C is $\sim 40\%$, for CS-C 20%, and CS-B is only very slowly degraded (12). Binding of all three CS types to NQ-BVH^{WT}, as well as to NQ-BVH^{ΔL} was evaluated accordingly (supplemental Figs. 1–6). Analysis of K_{DSUM} for

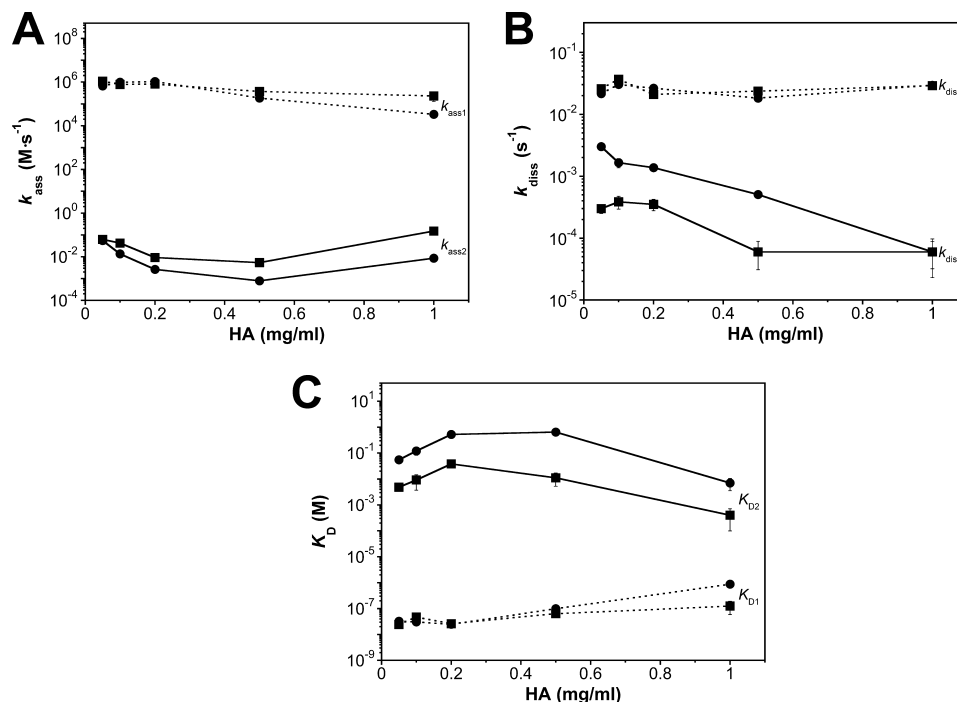


FIGURE 5. **Comparison of the association and dissociation rate constants of HA binding.** The association (k_a) (A) and the dissociation rate constants (k_{diss}) (B) of the first (filled circles and dotted line) and the second binding sites (filled circles and solid line) of NQ-BVH^{WT} and the first (filled squares and dotted line) and the second binding sites (filled squares and solid line) of NQ-BVH^{ΔL} were estimated directly during the fitting procedure. The K_D values of the first (filled circles and dotted line) and the second binding sites (filled circles and solid line) of NQ-BVH^{WT} and the first (filled squares and dotted line) and the second binding sites (filled squares and solid line) of NQ-BVH^{ΔL} were calculated from the association and the dissociation constants ($K_D = k_{\text{diss}}/k_a$) (C). Values represent the mean \pm S.E., and in 78% of all data sets, S.E. were $<25\%$.

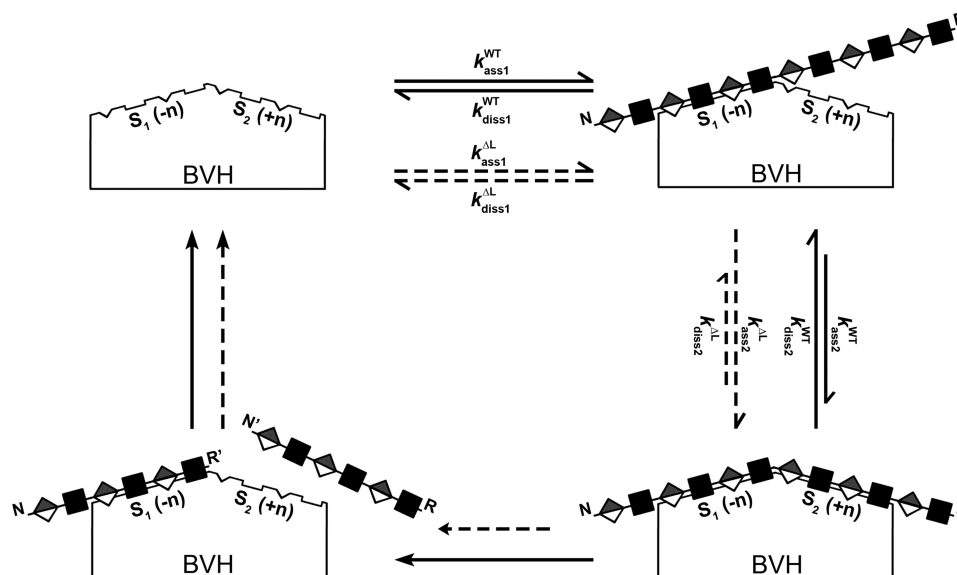


FIGURE 6. **Cleavage cycle of bee hyaluronidase.** The schematic representation of the model for the cleavage cycle was built based on HA binding and hydrolysis data. HA sugar residues are drawn according to commonly accepted graphical carbohydrate symbolism (49); *N*-acetyl-D-glucosamine is depicted as a filled square with solid lines, and D-glucuronic acid is depicted as a diamond with gray upper segment and black outline. Reducing ends of the substrate are indicated by *R*, non-reducing ends are indicated by *N*; in hydrolysis, products *R'* and *N'* are used accordingly.

the CS types with regard to HA showed that substrate binding is an important actuating value determining turnover rates (supplemental Table 1–3), as CS-B showed the highest K_{DSUM} value of 801 nM at 0.5 mg/ml for the wild-type and 257 nM at 0.2 mg/ml for the loop variant (supplemental Table 2), and CS-A and CS-C showed K_{DSUM} values of 172 nM at 0.5 mg/ml and 198 nM at 0.05 mg/ml for NQ-BVH^{WT}, and 42.5 nM at 0.2 mg/ml and 19 nM at 0.1 mg/ml for NQ-BVH^{ΔL}, respectively. Further kinetic analyses

showed best fit of the respective data again to the sequential two-site model. In view of these results, we propose here a model for the cleavage cycle of HAase featuring two distinct binding sites, which sequentially bind and release the products accordingly (Fig. 6).

DISCUSSION

The published crystal structures of HAase from bee (15) and wasp venom (31) or that of human serum HAase, human serum

hyaluronidase 1 (32) led to the identification of a bulky knob, which rises at the upper end of the substrate binding groove. Both length and amino acid composition of this unshaped bump are highly variable, with the sole exception of a single asparagine residue which is conserved in most species. In many hyaluronidases from hymenoptera, that asparagine is part of a potential *N*-glycosylation recognition sequence, which in all cases characterized so far is followed by a proline. This combination, however, renders this particular site highly unlikely for posttranslational glycosylation (33). This assumption was further strengthened by comparing wild-type BVH with BVH^{ΔL} in which the respective stretch had been replaced with a tetrapeptide sequence comprising alternating Gly and Ser residues. The latter thus lacks the potential glycosylation site. After expression in *Pichia pastoris*, a microbe known to efficiently glycosylate, the molecular weight of the two secreted proteins was found comparable. Interestingly, enzymatic and kinetic analyses of BVH^{ΔL} in which the blocking knob has been depleted showed that it has reduced activities.

Previous analyses with bovine testicular hyaluronidase had shown that two phases of HA hydrolysis can be distinguished: an early part, where high molecular weight HA was found to be degraded at a relatively fast rate; thereafter, the resulting oligomeric fragments were hydrolyzed with slow kinetics (26, 34, 35). This was found to be true for wild-type BVH activity. However, the results obtained for BVH^{ΔL}, which supposedly should offer more open space for a high molecular weight substrate, were unexpected. The rapid early phase of HA hydrolysis was not found for the enzyme variant. Although counterintuitive, this bulky loop actually enhances turnover of polymeric HA.

The initial reaction rates for both wild-type and BVH^{ΔL} clearly showed an inhibition at high HA concentration. As reported previously, short HA fragments are poor substrates for HAases (34, 35). Provided that the atypical bell-shaped progression of the substrate concentration dependence could be due to increasing amounts of short HA oligomers in concentrated polydispers substrate solutions. Increasing concentrations of short HA may thus shift to late phase kinetics and greatly decelerate the overall turnover rate. It is, however, also conceivable that similar to steric exclusion of bovine serum albumin in highly concentrated HA solutions (36, 37), the observed suppression of enzyme activity could also be a consequence of crowding out of HAase by closely interacting HA chains.

To address this question, the kinetic data sets were analyzed further. Working along these lines yielded only an approximation of Michaelis-Menten parameters. However, acceptable estimates for V_{\max} and K_m could still be achieved. An important result was that K_m for BVH^{ΔL} was found to be much lower, thus indicating enhanced affinity for HA.

More detailed binding studies were performed by means of QCM technology, which is capable of monitoring the interaction in real-time as a change in frequency of a surface-coated quartz crystal resonator. In solution, this method is highly effective at determining the affinity of molecules, in particular for large binding partners, such as high molecular weight HA. So far, no experimental results based on QCM were available for HA-HAase interaction. Surface plasmon resonance has previously been introduced to monitor the interactions of bovine

testicular hyaluronidase with sulfated glucosaminoglycans. Notably, however, HA as the major binding partner for HAase has for unknown reasons not been studied (38).

When using enzymatically active HAase in QCM measurements, binding could be examined, but due to hydrolysis taking place at the same time with concomitant generation of highly polydisperse HA, no distinct models for subsequent data analysis could be built. Stable interaction of HA, and other substrates such as CS with the enzyme could only be studied for HAase lacking hydrolysis activity. Mutations of specific catalytic residues in the active center were previously reported to inactivate human testicular hyaluronidase (24), as well as human serum hyaluronidase 1 (39). Point mutations introduced into BVH also yielded an inactive variant, which still bound high molecular weight substrate efficiently. From data obtained with this variant, a sequential two-site model could be deduced that features a dynamic mechanism involving a second potential binding site, which only becomes available after a first site has been successfully occupied by an uncleaved polymeric substrate. According to this model, we propose the existence of two kinetically distinct binding sites, which are both located in the substrate binding groove on either side of the active center. Following the nomenclature for sugar binding sites in glycosyl hydrolases (40), the first binding site (S1) is located adjacent to the non-reducing end away from the point of cleavage and therefore labeled as the $-n$ subsites, whereas the second binding site (S2), which we mapped next to the bulky formation interacts with HA at $+n$ subsites, with cleavage taking place in between -1 and $+1$.

The model of the cleavage cycle as depicted in Fig. 6 states that initial attachment at S1 is mandatory for HA to subsequently interact with S2. After completion of the hydrolytic reaction the HA fragment bound to S2 will be released first, and only thereafter the remaining fragment detaches from S1. Replacement of the loop yielded a variant with lower enzymatic activity but with up to 100 times higher affinity in HA-binding. The equilibrium dissociation constant, K_D for high molecular weight HA was found to be 0.05 to 0.98 nM, whereas the K_D for the wild-type enzyme was only 1.77 to 62.9 nM. Previously reported K_D for HA-binding proteins or hyalactins were 5–150 μ M for CD44 (41–43), 11–36 μ M for LYVE-1 (43), 0.2–55 μ M for TSG-6 (44, 45), 226 nM for aggrecan, and exactly 82 nM for Link protein (46). The HA receptor for endocytosis has the highest affinity for HA ranging 5 to 23 nM (47, 48). Compared with these specific binding proteins, the BVH loop variant shows the highest avidity for HA.

Indeed, the bulky formation interferes with HA binding but probably promotes the unbinding of short oligomeric chains tethered to the $+n$ site. The penultimate step of the catalytic cycle had actually been depicted before by x-ray structure determination of BVH co-crystallized with an HA hexasaccharide. In the crystals, the HA hexasaccharide had been degraded, and the HAase only retained a tetrasaccharide fragment bound to the subsites -4 to -1 . At the $-n$ subsites amino acids, which directly interact with HA such as Tyr-55, Asp-111, Glu-113, Tyr-184, Tyr-227, Trp-301, Ser-303, and Ser-304 could be identified (15). In turn, however, the situation regarding the $+n$ subsites was less clear. To gain more insight, the three-dimen-

sional coordinates of HA derived from the crystal structure of the BVH-HA complex were used for computational modeling studies placing a virtual HA chain all along into the binding groove of HYAL1. In this way amino acid residues located as close as 5 Å to the HA molecule could be identified. These were Asp-129, Glu-131, Tyr-202, Tyr-247, Ser-245, and Arg-265 (39). The latter two correspond to Ser-225 and Arg-244 of the bee enzyme. However, no direct interaction with the HA tetrasaccharide had been reported in the previous study (15). Also in close vicinity to the bulky knob, Ser-225 and Arg-244 are likely residues of the +*n* subsites and thus shape the second binding site.

In conclusion, we report here that the bulky formation, which at first glance appears to block the substrate binding groove, actually contributes to a second HA binding site in HAase. The results presented in this study demonstrate that this loop is involved in modulation of the enzymatic activity, which besides substrate binding also requires efficient clearing of the products after catalytic processing. In turn, when a product is transiently retained at the second site through strong binding, further progression through the catalytic cycle is retarded. Through the bulky loop in BVH unbinding and release of the cleaved HA fragment from the enzyme is enhanced, altogether resulting in a higher rate of hydrolysis.

The various bulky formations in different hyaluronidase species may have evolved to efficiently hydrolyze high molecular weight HA in different biological processes. The bee venom enzyme is considered to be a spreading factor. At the site of a bee sting, rapid hydrolysis of HA chains in the dermis facilitates the diffusion of other venom constituents, mainly the lytic peptide mellitin and phospholipase, thereby augmenting local damage and inflammation.

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